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APPLICATION

FOR

UNITED STATES LETTERS PATENT

APPLICANT : Michael E. Mendelsohn
TITLE : METHOD FOR ASSAYING COMPOUNDS AFFECTING CELL DIVISION

PATENT
ATTORNEY DOCKET NO.: 00398/506001

METHOD FOR ASSAYING COMPOUNDS AFFECTING CELL DIVISION

Statement as to Federally Sponsored Research

The invention was made, in part, with funding from the National Institutes of Health, grant HL56069. The government may have certain rights in the invention.

BACKGROUND OF THE INVENTION

Estrogen receptor beta (ER beta), first reported in 1996, is the second estrogen receptor cloned. Like the original estrogen receptor (ER alpha), ER beta has the ability to act as a transcription factor by binding estrogen. The amino-terminal domain of ER beta differs considerably from that of ER alpha, while the DNA binding domain is highly homologous, and the hormone binding and carboxyl-terminal domains are partially homologous, to ER alpha.

While ER beta, like ER alpha, binds hormone and modulates changes in transcription, a fundamental question has been whether ER beta possesses additional function(s). ER beta has been postulated to play a role in the cardiovascular system and may be involved in the vascular protective effects of estrogen (Iafrati et al., Nat. Med.

3:545-8, 1997). An estrogen receptor alpha knock-out mouse was still protected against vascular injury upon estrogen administration. This implied another way for estrogen to exert its protective effects and led to the discovery of ER beta in blood vessels. ER beta was discovered in the rat prostate, originally, and was then found to be expressed in blood vessels, including the blood vessels of the ER knock-out mouse. Furthermore, if a blood vessel of a male rat is injured, regrowth of endothelial and smooth muscle cells occurs as that injury repairs itself, accompanied by a dramatic increase in ER beta expression at the leading edge of cells as they grow back. There is no change in the expression of ER alpha in this setting (Lindner et al., Circ. Res. 83:224-9, 1998).

SUMMARY OF THE INVENTION

I have discovered, unexpectedly, that ER beta interacts with Mitosis Arrest Deficient 2 (MAD2), a cell cycle checkpoint protein. Accordingly, the invention features a novel method for assaying compounds that affect cell division by determining the interaction between estrogen receptor (ER beta) and MAD2

In more detail, the invention features a method for determining whether a test compound is potentially capable of affecting cell division, by: a) contacting the test compound with ER beta and MAD2, or binding fragments thereof, under conditions in which ER beta and MAD2 or fragments have formed, or are able to form, a complex; and b) determining whether the test compound affects the ER beta/MAD2 complex or

complex formation, as an indication that the test compound is potentially capable of affecting cell division.

In preferred embodiments of the invention, the assay may be conducted *in vitro* or *in vivo*. In other preferred embodiments of the invention, a yeast two-hybrid system, a GST-fusion protein interaction assay, fluorescence spectroscopy, or biomolecular interaction analysis using, for example, chip technology, may be used to assay the test compound.

By "test compound" is meant any chemical compound, be it naturally-occurring or artificially-derived. Test compounds may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, and nucleic acid molecules.

By "affecting" is meant changing, either by increase or decrease.

By "cell division" is meant a process by which a cell replicates itself. As defined in this invention, cell division may include any stage in the cell cycle, such as mitosis, G_1, S phase, and G_2.

By "determining" is meant analyzing the effect of a test compound on the test system. The readout of the analysis may be disruption or enhancement of the interaction between ER beta and MAD2, as well as measures of cell division. The means of analyzing may include, for example, yeast two-hybrid assays, GST fusion protein interaction, immunoprecipitation, fluorescence spectroscopy, biomolecular interaction

analysis, and other methods known to those skilled in the art.

The invention provides a means of identifying test compounds that affect cell division. This is particularly useful since abnormalities in cell division have been associated with a variety of conditions associated with cell proliferative diseases. Thus, compounds that affect cell proliferation may be used in therapy, prevention, or diagnosis of such diseases.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A Interaction of mERβ with EC1 in yeast.

Fig. 1B Interaction of hERα with EC1 in yeast.

Fig. 2A Alignment of nucleotide sequences of EC1 (SEQ ID NO:3) and hsMAD2 (SEQ ID NO:1).

Fig. 2B Alignment of amino acid sequences of EC1 (SEQ ID NO:4) and hsMAD2 (SEQ ID NO:2).

Fig. 3A Schematic diagram of mERβ cDNA showing domains and the mERβ mutants that were constructed to correspond to the domains.

Fig. 3B Summary of HIS and β-Galactosidase reporter activation for the mERβ/GalBD and mERβ/GalAD mutants cotransformed in Y190 with EC1/GalAD or EC1/GalBD respectively.

Fig. 4A GST-fusion protein interaction studies with mERβ and EC1.

Fig. 4B GST-fusion protein interaction studies with MAD2 and mERβ.

Fig. 4C GST-fusion protein interaction studies with hERα and EC1.

Fig. 4D GST-fusion protein interaction studies with MAD2 and mERβ mutants.

DETAILED DESCRIPTION OF THE INVENTION

I have discovered a novel interaction between the estrogen receptor ER beta and a protein called MAD2, which is a cell cycle checkpoint protein. This is a surprising finding and has interesting scientific and practical implications.

Function of the MAD2/ER beta Interaction

To address the question as to the function of the ER beta/MAD2 interaction, one can begin with an overview of the information previously known about MAD2. MAD2 is a member of a family of proteins that are involved in cell surveillance of the spindle assembly checkpoint during cell division (Elledge, Science 279:999-1000, 1998). The checkpoint blocks separation of sister chromatids in the dividing cell until the two sister chromatids are attached to the opposite poles of the spindle. Unless each sister chromatid is aligned correctly through its link to its connecticore, cell division will not take place. On page 1000 of the Science article, Elledge states: "Although pleasing, this model contains several unresolved but important details. For example, is the spindle checkpoint transiently activated in every cell cycle or only in response to spindle perturbation?"

Confocal immunofluorescence microscopy shows that ER beta is localized with

MAD2 at the kinetochore when the cell is in prometaphase. Therefore, ER beta is part of the spindle checkpoint and the ER beta/MAD2 complex may play a physiological role in every cell during the cell cycle. This confirms other protein-protein interaction data shown herein and also reveals the presence of ER beta in the kinetochore, a discovery which is totally unexpected and is the first instance of the localization of a steroid hormone receptor to the cell division molecular apparatus. The results presented herein indicate that the interaction between ER beta and MAD2 affects cell division.

Drug Screening

As is stated in the Summary of the Invention, above, compounds that affect the interaction of ER beta with MAD2 are potential cell cycle-affecting drugs.

Cell-based Assays

One method for screening test compounds can employ a cellular system, in which the components are present inside a cell. The test compound, at concentrations of approximately 10^{-12} -10^{-5} M, is also placed in the cell, and interacts with, or fails to interact with, ER beta and MAD2 in the cellular environment. Yeast and mammalian two hybrid systems are examples of such systems, and are commercially available (Clontech, Palo Alto, CA; Promega, Madison, WI) or can be readily developed to suit specific screening applications. Cellular systems have the potential advantage that other molecules, present in a living cell, might participate in the interaction. Test compounds

that affect the interaction between ER beta and MAD2 in such systems, therefore, are more likely to be of physiological relevance.

Non-cell-based Assays

One straightforward readout for the effect of a test compound on the ER beta/ MAD2 interaction is to employ an *in vitro* system in which one or both of ER beta or MAD2 is linked to a fluorophore that only fluoresces, or fluoresces at a different wavelength, if ER beta and MAD2 interact with each other. Thus, fluorescence at a given wavelength occurs only if the conformation of one of the pair is changed because of its interaction with the other member of the pair; one can therefore determine, by detecting the intensity or wavelength of the fluorescence, the extent to which the test compound influenced, or failed to influence, the conformational change that accompanies complex formation. Fluorophores may include, for example, synthetic compounds or proteins such as green or blue fluorescent proteins or phycobiliproteins.

Another non-cell based system for the effect of a test compound on the ER beta/ MAD2 interaction is the biomolecular interaction analysis (BIA) system, which examines the interaction of molecules in real time. BIA is an affinity-based biosensor technology that relies on the optical phenomenon of surface plasmon resonance (SPR), which detected changes in the refractive index of a solution close to the surface of a sensor chip. Either one of ER beta or MAD2 can be immobilized in a suitable matrix on the sensor chip, which forms one wall of a micro-flow cell. Sample, such as cell extract containing

the other interactant (MAD2 or ER beta), and a test compound can then be injected over the surface in a controlled flow. Changes in surface concentration resulting from a change in the interaction between MAD2 and ER beta is detected as a SPR signal which is expressed in resonance units. An advantage of BIA technology is that complex interactions, involving multimeric complexes, may be examined. The concentrations of the test compounds may range from approximately 10^{-12} -10^{-5} M.

Therapeutic Methods

The invention contemplates two broad categories of therapeutic methods based on the present discovery: administration of compounds (drugs) identified using the above-described screening methods, and gene therapy using DNA encoding ER beta or MAD2, or fragments of one of these proteins. ER beta/MAD2-based therapies identified according to the present invention may be used alone, or as an adjunct to other therapies, such as, for example, with vascular endothelial growth factor for therapeutic angiogenesis.

Drug Treatment

Two main classes of drugs can be identified by the above-described screening methods: 1) drugs that inhibit the binding of ER beta to MAD2, and 2) drugs that promote or enhance such binding.

Enhancement of the ER beta/MAD2 Interaction

Assuming that formation of the ER beta/MAD2 complex acts, *in vivo*, as a brake on the cell cycle; i.e., that the interaction slows cell proliferation, drugs that promote the formation of the complex are potentially useful in the treatment of any of the many known hyperproliferative disorders, including cancer, psoriasis, excessive scar tissue formation, atherosclerosis, restenosis following vascular injury, and adhesion formation. Such anti-proliferative drugs are administered by any of the routes by which other known anti-proliferatives are administered; e.g., orally or intravenously, admixed with any appropriate pharmaceutically acceptable carrier. In some cases, such as widely diffused cancer, systemic administration will be preferred. In other instances, e.g., to inhibit vein or artery graft restenosis or to treat a site-confined solid tumor, the drug will be delivered locally, to the site of hyperproliferation, by any appropriate means, e.g., direct injection or delivery via catheter. When the site of hyperproliferation is the skin, e.g., in the case of psoriasis, the drug can be applied topically in the form of a cream or ointment. Should pulmonary administration be required, the drug can be administered by aerosol.

Promotion of complex formation that slows cell growth might also treat cardiovascular disease, because the interaction might "time" the repair process in the vessel, since ER beta is up-regulated during the repair process. Further, with respect to cancer, the role of ER beta in interacting with MAD2 may be to increase the fidelity and sensitivity of MAD2's surveillance for aberrantly aligned chromatids and for cells that are

aberrant in their chromosomal segregation. Thus, ER beta would be up-regulated in times of rapid proliferation, to eliminate aberrant cells that arise during such an increase in proliferation.

Inhibition of the ER beta/MAD2 Interaction

Assuming, still, that the formation of the ER beta/MAD2 complex acts, *in vivo*, as a brake on the cell cycle, drugs that interfere with, or block the formation of, the complex are potentially useful in the treatment of any of the known medical condition in which increased cell growth is desired. Examples are wound healing; vascular regrowth or angiogenesis following a myocardial infarction or other disorders in which tissues are underperfused; and hepatic regrowth following transplant of a piece of donor liver. Administration is as described above for complex-promoting drugs.

With respect to the possibility that the ER beta/MAD2 interaction facilitates, rather than slows, cell division, this is less likely given the present data since the ER beta knock-out mouse remains well protected by estrogen. If the complex enhanced cell proliferation, one would predict less of an injury in the ER beta knockout animal, and in fact we see the same level of injury and the same degree of protection by estrogen. Nonetheless, enhancement of cell proliferation by the complex cannot be ruled out, and if it is the case, promotion of complex formation is potentially a therapeutic approach to the treatment of diseases, e.g. cardiovascular diseases and wound-healing, in which such

enhanced cellular proliferation is desired. Conversely, inhibition of complex formation would provide a therapeutic approach to the treatment of hyperproliferative disorders such as cancer and psoriasis.

Nucleic Acid Therapy

Just as drugs (generally, small organic molecules) identified in the screening assays of the invention can either act as promotors or inhibitors of the ER beta/MAD2 complex, nucleic acid molecules can serve either of those two functions.

Several nucleic acid-based strategies can be employed to enhance or interfere with the ER beta/MAD2 interaction. All of these approaches can employ any of the known methodologies for delivering nucleic acids (usually DNA) to cells and tissues. These include the use of viral vectors, derived, for example, from retroviruses, adenoviruses, adeno-associated viruses, and lentiviruses, plasmids packaged in liposomes or linked to cationic polymers, and the use of naked i.e.,uncomplexed, charged DNA.

Enhancement of the ER beta/MAD2 Interaction

Several classes of nucleic acid molecules can enhance ER beta/MAD2 complex formation, ultimately resulting in an increased amount of the complex within cells, or in the formation of tighter complexes (shifting the complex formation to the right, favoring complex formation over dissociation in the *in vivo* equilibrium).

The most straightforward nucleic acid-based strategy for increasing the amount of ER beta/MAD2 complex is to provide expressible nucleic acid encoding one (or both) of

the proteins. For example, in some clinical situations the factor that limits the amount of complex present is an inadequate amount of one of the two proteins; that protein is, thus, supplemented by providing to the cells nucleic acid encoding it. As is described above, the expressible DNA is provided using any of the known, available methods, e.g., retroviral or other viral vectors.

It is also possible that a biologically active complex can be formed from the ER beta and MAD2 that are not full-length. Thus, nucleic acid encoding the binding regions of these proteins can be used in the complex-enhancing methods of the invention.

Inhibition of the ER beta/MAD2 Interaction

As is mentioned above, some medical conditions may be treated by inhibiting the complex formation of ER beta with MAD2. This can be accomplished by administration of a nucleic acid molecule encoding a fragment of one of the proteins that is capable of binding to the other protein, or of a sequence closely related to the binding domain that disrupts the interaction, thus competitively inhibiting the undesired protein-protein interaction. The use of anti-sense RNA is also contemplated as an approach that can interfere with the ER beta/MAD2 interaction.

Diagnostic Applications

Mutants or allelic variants of ER beta or MAD2 that have an altered level of interaction with the other are likely to exist. Accordingly, a screening method to identify people who are more or less susceptible to certain hyperproliferative diseases can be

developed. Thus, for example, there may be men who develop prostate cancer because they have an allelic variant of ER beta with an altered binding domain for MAD2, rendering the interaction between the two less favorable or less tight. That variation would be easily screened for, to detect men at higher risk for prostate cancer. Conversely there might be men who have allelic variants that provide greater protection from prostate cancer.

Examples

Yeast two-hybrid assays

Library screening

The full-length coding region of mouse ER beta (mERβ) was cloned in frame into the EcoRI site of the Gal4 DNA binding domain (BD) vector, pGBT9, to yield the construct mERβ/BD. mERβ/BD and an ovine pulmonary artery endothelial cell (immortalized, P11) library (constructed using the Two-hybrid cDNA Library Construction Kit, Clontech, Palo Alto, CA, USA), cloned into the Gal4 DNA activating domain (AD) vector pGAD10, were transformed into *Saccharomyces cerevisiae* strain Y190 (MAT a, ura3-re, his3-200, lys2-801, ade2-101, trp1-901, leu2-3112, gal4Δ, gal80Δ, cyhr2, LYS2::GAL1UAS-HIS2TATA-HIS2, URA3::GAL1UAS-GAL1TATA-lacZ; Clontech, Palo Alto, CA) using the lithium acetate method.

Polypeptides interacting with mERβ/BD were detected by their ability to reconstitute the GAL4 transcription factor and activate transcription of HIS3 and LacZ

reporter genes. *S. cerevisiae* colonies able to grow on media lacking histidine were assayed for β-galactosidase activity using a colony lift filter assay.

Colonies were lifted onto MSI NitroPure nitrocellulose filters (MSI, Westoboro, MA, USA) which were then submerged in liquid nitrogen for 15 seconds, placed on Whatman #5 paper presoaked in Z-buffer (60mM Na2HPO4.7H2O, 40mM NaH2PO4.H2O, 10mM Kcl, 1mM MgSO4, 7H2O, 50mM β-mercaptoethanol, pH7.0), and incubated at 37° for up to 12 hours. PGAD10 plasmids from His+, LacZ+ colonies were isolated and the library cDNA insert sequenced. Four double transformation individual plasmids were reintroduced into *S. cerevisiae* strain Y190 by the lithium acetate method, and reporter activation assayed as above.

Screening of the ovine library yielded clone EC1. Fig. 1 shows the definitive interaction between the binding domain of ER beta and clone EC1. Yeast strain Y190 was transformed with mERβ/BD in combination with EC1/AD and GalAD respectively and plated on Sc-His (synthetic complete medium, minus histidine) and YPD (yeast extract, peptone, dextrose) plates (Fig. 1A). Positive interaction of mERβ/BD with EC1/AD was seen in the presence, as well as in the absence, of estrogen. Yeast strain Y190 was also transformed with human ER alpha (hERα)/BD in combination with EC1/AD, hERα/BD and Trip 1/AD and plated on Sc-His and YPD plates. Positive interaction of hERα/BD with hERα/GalBD and Trip1/GalAD was seen only in the presence of estrogen. Colony lift β-Galactosidase assays were also performed with yeasts

from the YPD plate in both Fig. 1A and 1B. TIF2 and TRIP1 homologs were found previously by screening a human heart library with mERβ as bait. Thus, these results indicate that the interaction between EC1 and ER beta is estrogen independent, while that between EC1 and ER alpha is estrogen dependent. This implies a new function for ER beta that may be separated from its estrogen binding function.

EXAMPLE 2

The ER-beta/MAD2 Interaction

Sequencing of clone EC1 revealed that it was almost identical to a well-known and well-studied cDNA called MAD2 (Fig. 2). Alignment of the EC1 nucleotide sequence (SEQ ID NO:3) with that of *homo sapiens* MAD2 (hsMAD2) (SEQ ID NO:1) revealed 93% identity. Identical nucleotides are marked with asterisks (Fig. 2A). Similarly, alignment of the amino acid sequence of EC1 (SEQ ID NO:4) with that of hsMAD2 (SEQ ID NO:2) revealed 95% identity. Identities are shown by the abbreviation of the corresponding amino acid, similarities by + symbols, and amino acid mismatch by a gap (Fig. 2B). The difference between EC1 and MAD2 is likely to be due to inter-species variation because EC1 was obtained from sheep and the particular MAD2 that was used for the sequence comparison was of human origin.

Experiments that showed that ER beta and MAD2 form complexes with one another were then carried out. These results were unexpected; MAD2 would not have been expected to interact with ER beta based on any prior knowledge about either protein.

Fig. 3 is a schematic diagram summarizing the MAD2/ER beta interaction. A series of truncations were made and tested in the two hybrid system to determine whether ER beta/MAD2 interactions are maintained or lost with a given deletion.

The N-terminal mERβ truncation mutants, mERβ 1-622 and 1-516, were constructed by digestion with Sma1 or BamH1 respectively. Following restriction enzyme digests, mERB was religated in pGBT9 and reintroduced into yeast strain Y190. A C-terminal mERβ truncation mutant was constructed by excising the BamH1 mERβ fragment in order to get mERβ 516-1458. mERβ, following restriction enzyme digestion, was religated in pGAD10 and reintroduced into yeast strain Y190. To create a mERβ 516-641 mutant, EcoR1 and BamH1 sites were engineered at amino acids 172 and 213, respectively, with PCR primers. The resulting PCR product was ligated into pGBT9 and reintroduced into yeast strain Y190. The correct reading frame was verified by DNA sequencing. An N-terminal EC1 truncation mutant was constructed by excising the EcoR1 EC1 fragment for EC1 1-~1900. Again, following restriction enzyme digest, EC1 was self-ligated in PGBT0 and reintroduced into Y190. Expression of mERβ1-516 in pGBT9 was confirmed by a Western blot of the Y190 lysate using the rabbit polyclonal Gal4 DNA binding domain antibody (Upstate Biotechnology, Lake Placid, New York).

Assay of the truncation mutants proved to be a sensitive and specific screen for the identification of the MAD2/ER beta interaction domain. The interaction domain was identified as encompassing amino acids 516 to 622 (Fig. 3A). Fig. 3B summarizes the

two hybrid protein interaction results. As is shown in Fig. 3B, the ER beta/MAD2 interaction domain is defined by amino acids 516 to 641 of clone EC1. Fig. 3B also shows that slightly larger regions, containing the interaction domain, support the interaction between ER beta and MAD2, while fragments lacking the 516-622 amino acid domain do not.

GST-pull down assays

GST-fusion protein interaction assays were also performed to examine the interaction between ER beta and MAD2.

EC1 1-~1900, the N-terminal mERβ trunction mutant mERβ1-622 (EcoR1/SmaI), and the C-terminal mERβ truncation mutant mERβ516-1458 (BamH1/EcoR1) were ligated into PGEX-4T-1 (Pharmacia Biotech, Piscataway, NJ). The mERβ 518-622 mutant was constructed by excising the BamH1 mERβ1-622 fragment. Following restriction enzyme digest, mERβ was religated into PGEX-4T-1. Full length ratERβ ligated into PGEX-2TK was obtained from Dr. Myles A. Brown (Dana Farber Cancer Institute, Boston, MA). GST-mERβ-622, GST-mERβ516-1458, GST-mERβ516-622 and GST-rERβ were used to express GST fusion proteins as follows.

Fresh overnight cultures of *Eschericia coli* (XL10-Gold: Stratagene; BL21: Novagen) transformed with one of the pGEX-4T-1 or pGEX-2TK recombinants were diluted 1:10 in Luria-Bertoni medium (LB) containing ampicillin (100 mg/ml) and

incubated at 37°C with shaking. After 1 hour of growth, isopropyl-β-D-thiogalactopyranoside (IPTG, Calbiochem-Novabiochem, LaJolla, CA) was added to a final concentration of 0.1 mM.

For analysis of total bacterial protein content, aliquots of each bacterial culture were pelleted in a microcentrifuge, boiled in SDS sample buffer (10% Glycerol, 50mM Tris pH 6.8, 1% SDS, 5% β-mercaptoethanol, 0.01% Bromophenol Blue), and loaded onto an SDS-polyacrylamide gel. Proteins were visualized by Coomassie blue staining. The GST fusion proteins were immobilized on Glutathione Sepharose beads.

Recombinant [^{35}S]mERβ, [^{35}S]hERα and [^{35}S]hsMAD2 were produced by using *in vitro* transcription/translation assay and [^{35}S]methionine for labeling (TNT Coupled Reticulocyte Lysate Systems kit, Promega, Madison, WI, USA). CMV3mERβ-ZEO, CMV3ERα and pET28a(+)hsMAD2 (Dr. R. Benezra, The Memorial Sloan Kettering Cancer Center, New York, NY) were used as templates, respectively.

Recombinant proteins were incubated at 4° in suspension buffer (20mM Tris pH7.4, 137mM NaCl, 2mM EDTA pH7.4, 1% TritonX-100, 10% Glycerol, 25 mM β-glycerolphosphate, 1mM Na vanadate, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 1mM PMSF) with either one of the GST fusion proteins immobilized on glutathione-Sepharose beads for 1 hour. Beads were then washed three times in suspension buffer, boiled 5 minutes in SDS sample buffer, and associated proteins resolved by SDS-PAGE. Labeled protein bands in gels were then visualized by autoradiography.

For fusion protein recovery using glutathione-Sepharose (Pharmacia, Piscataway, NJ), bacterial cultures were pelleted by centrifugation at 5000 x g for 5 min at 4°C and resuspended in 1/10 volume NETN (20 mM Tris pH 8.0, 100mM NaCl, 1mM EDTA, 0.5% NP-40). The bacteria were then lysed on ice by mild sonication and centifuged at 10.000 x g for 5 min at 4° C. The bacterial supernatant (~10ml) was rocked for 2 hrs at 4°C with 1 ml of glutathione-Sepharose which had been previously washed three times and resuspended (final concentration 1:1 v/v in NETN). The glutathione-Sepharose beads were then washed three times in NETN. For analysis of bound proteins, the beads were boiled in 1x sample buffer (10% Glycerol, 50mM Tris pH 6.8, 1% SDS, 5% β-mercaptoethanol, 0.01% BromphenolBlue) and loaded onto SDS-polyacrylaminde gels. Proteins were visualized by Coomassie blue staining.

Fig. 4 shows the GST-fusion protein results. [^{35}S] labeled mERβ was incubated with GST-beads (Lane 2) and GST-EC1 beads (Lane 3) followed by SDS-PAGE and autoradiography. Lane 1 shows 10% of the [^{35}S]mERβ input (Fig. 4A). In the complementary experiment (Fig. 4B), [^{35}S]-labeled hsMAD2 was incubated with GST beads (Lane 2) and GST-mERβ beads (Lane 3) followed by SDS-PAGE and autoradiography. Lane 1 shows 15% of [^{35}S] MAD2 input (Fig. 4B). GST-fusion protein interaction studies were also done with hERα. [^{35}S] labeled hERα was incubated with GST-mERβ (Lane 2, positive control), GST-beads (Lane 3) and GST-EC1 beads followed by SDS-PAGE and autoradiography. Lane 1 shows 5% of the [^{35}S] hERα input

(Fig. 4C). Fig. 4D shows GST-fusion protein experiments with [^{35}S] labeled MAD2 and mERβ mutants. [^{35}S]MAD2 was incubated with GST-beads (Lane 2), GST-mERβ 516-622 beads (Lane 3), GST-mERβ 1-622 beads (Lane 4), GST-mERβ 516-1458 beads (Lane 5) and GST-mERβ beads (Lane 6), followed by SDS-PAGE and autoradiography. Lane 1 shows 5% of [^{35}S]hsMAD2.

Thus the GST-fusion protein experiments demonstrate that, mERβ is brought down, or associates with, the GST-MAD2 clone and, in the converse experiment, MAD2 is brought down by GST-mERβ. Each case demonstrates the protein-protein interaction. In contrast, the results shown in Fig. 4C indicate that while GST-mERβ, as expected, brings down ERα alpha (this is a positive control since it is known that these two proteins heterodimerize), GST alone, or GST MAD2, shown in the third and fourth lanes, respectively, do not bring down ER alpha. This result confirms the two hybrid data, i.e. that ER alpha does not interact with MAD2. Fig. 4D, which shows the results of protein-protein interaction studies between MAD2 and ER beta mutants, also confirms the two hybrid data which identified the MAD2/ER beta interaction domain as including amino acids 516-622. Other experiments indicate that MAD2 does not interact with RAR or RXR (two steroid hormone families members), further underscoring the specificity of the MAD2/ER beta interaction.

PLASMIDS used for the above-described experiments

Two-hybrid system

mERβ/GalBD + GalAD

Made from pCRII-mERβ (short variant, complete ORF 1-1458) by using EcoR1 as the restriction site.

mERβ1-622/GalBD

Cloned by digesting mERβ/GalBD with Sma1, purification and subsequent re-ligation.

mERβ1-516/GalBD

Cloned by digesting mERβ/GalBD with BamH1, purification and subsequent re-ligation.

mERβ516-641/GalBD

Engineered by PCR (5'primer: EcoR1-site, 3'primer:BamH1-site) with mERβ/GalBD as template and cloned into pGBT9.

mERβ516/1458/GalAD

Cloned by digesting mERβ/GalAD with BAMH1, purification and subsequent re-ligation.

ERα/GalBD + GalAD

Made from pHEGO (human estrogen receptor alpha) by using EcoR1 as the restriction site.

TIF2/GalAD

Obtained from 2-hybrid screening (human heart library) with mERβ as bait (C9).

TRIP1/GalAD

Obtained from 2-hybrid screening (human brain library) with mERβ as bait (Br5).

GST-pull down assay

pGEX-EC1

The N-terminal part of EC1/GalAD (~1.9bp) was excised by EcoR1 and cloned into pGEX-4T-1. XL10-Gold was used for fusion protein expression.

pGEX-ratERβ

From Dr. M. Brown (Dana-Farber Cancer Institute, Boston, MA). ratErβ is cloned into pGEX-2TK.

pGEX-mERβ1-622

The N-terminal part of mERβ/GalBD was excised with EcoR1 and Sma1, purified and cloned into pGEX-4T-1. Fusion protein expression was performed in BL21.

pGEX-mERβ516-1458

The C-terminal part of mERβ/GalBD was excised with BamH1 and EcoR1, purified and cloned into pGEX-4T1. Fusion protein expression was performed in BL21.

pGEX-mERβ516-622

The mERβ516-622 mutant was constructed by excising the BamH1 mERβ1-622 fragment. Following restriction enzyme digestion, mERβ was religated into pGEX-4T-1. Fusion protein expression was performed in BL21.

In vitro transcription/translation assay

[^{35}S] hMAD2

DNA template: full-length hs MAD2 ORF was subcloned (EcoR1-in frame with his-tag/Not1) into pET-28A(+) and provided to us by R. Benezra (Science, 1996, **274**, 246-248)

[^{35}S] mERβ

DNA template: CMV3ERβ-ZEO

[^{35}S] hERα

DNA template: CMV3ER

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

What is claimed is: